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Oestrogenic activity of a textile industrial wastewater treatment plant effluent evaluated by the E-screen test and MELN gene-reporter luciferase assay

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(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

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Title: Estrogenic activity of a textile industrial wastewater treatment plant effluent evaluated by the E-screen test and MELN gene-reporter luciferase assay.

Article Type: Full Length Article

Section/Category: Biomonitoring and Surveillance

Keywords: Estrogenic activity; E-screen; MELN; wastewater treatment plant; textile; ozonation; cytotoxicity.

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First Author: Tiziana Schilirò, M.D.

Order of Authors: Tiziana Schilirò, M.D.; Arianna Porfido; Federica Spina; Giovanna Cristina Varese; Giorgio Gilli

Abstract: This study quantified the biological estrogenic activity in the effluent of a textile industrial wastewater treatment plant (IWWTP) in northwestern Italy. Samples of the IWWTP effluent were collected monthly, both before and after tertiary treatment (ozonation). After solid phase extraction, all samples were subjected to two in vitro tests of total estrogenic activity, the human breast cancer cell line (MCF-7 BUS) proliferation assay, or E-screen test, and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay, to measure the 17 β -estradiol equivalent quantity (EEQ). In the E-screen test, the mean EEQ values were 2.35 \pm 1.68 ng/L pre-ozonation and 0.72 \pm 0.58 ng/L post-ozonation; in the MELN gene-reporter luciferase assay, the mean EEQ values were 4.18 \pm 3.54 ng/L pre-ozonation and 2.53 \pm 2.48 ng/L post-ozonation. These results suggest that the post-ozonation IWWTP effluent had a lower estrogenic activity (simple paired t-tests, $p < 0.05$). The average reduction of estrogenic activity of IWWTP effluent after ozonation was 67 \pm 26 % and 52 \pm 27 % as measured by E-screen test and MELN gene-reporter luciferase assay, respectively. There was a positive and significant correlation between the two tests ($Rho S = 0.650$, $p = 0.022$). This study indicates that the environmental risk is low because estrogenic substances are deposited into the river via IWWTP at concentrations lower than those at which chronic exposure has been reported to affect the endocrine system of living organisms.

Response to Reviewers: Reviewer #7:

This is the revised version of this manuscript incorporating the reviewers' comments. The authors also include the item wise response to comments along with the manuscript. The study aims to quantify the biological estrogenic activity in the effluent of a textile industrial wastewater treatment plant (IWWTP) in northwestern Italy. Samples of the IWWTP effluent were collected monthly, both before and after tertiary treatment (ozonation). After solid phase extraction, all samples were subjected to two in vitro tests of total estrogenic activity, the human breast cancer cell line (MCF-7 BUS) proliferation assay, or

E-screen test, and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay, to measure the 17 β -estradiol equivalent quantity (EEQ). The study indicates that the environmental risk is low because estrogenic substances are deposited into the river via IWWTP at concentrations lower than those at which chronic exposure has been reported to affect the endocrine system of living organisms.

Overall, the manuscript has been revised well taking in to account the comments on the previous submission and seems to be a much improved document. The manuscript will benefit from some minor modification before publication.

Specific comments as follows:

1. QA/QC protocol and data: Any biological tests require an elaborate QA/QC protocol and data collection, this manuscript does not adequately provide the details of the same used in the study.
2. Table-1, replace "commas" used for the decimal places and the standard notation of "." to avoid confusion.
3. Table-2, for "nd", the information is not complete unless the detection limit is also provided.
4. Figure-1, the box and whisker plot needs details on what it showing (e.g., ranges showing X percentiles, the thick line in the box is the mean or the median etc.), these details can be provided as a legend in the figure or in manuscript text.

Response to Reviewer comments:

1. Thanks to reviewer comments we add and highlight the QA/QC at lines: 151-152, 194-200, 232-236, 280, 313-314.

We initially did not report about QA/QC protocol because the in vitro tests were performed by authors in other manuscripts (1. Schilirò T, et al. 2004. Toxicity and Estrogenic Activity of a Wastewater Treatment Plant in Northern Italy. *Arch Env Contam Tox* 47(4):456-462 - 2. Bicchi C, et al. 2009. Analysis of environmental endocrine disrupting chemicals using the E-screen method and stir bar sorptive extraction in wastewater treatment plant effluents. *Sci Total Env* 407:1842-1851 - 3. Schilirò T, et al. 2009. The endocrine disrupting activity of surface waters and of wastewater treatment plant effluents in relation to chlorination. *Chemosphere* 75:335-340 - 4. Schilirò T, et al. 2011. Endocrine disrupting activity in fruits and vegetables evaluated with the E-screen assay in relation to pesticide residues. *J Steroid Bioch Mol Bio* 127:139-146).

2. We replace "commas" in Table 1.

3. We provided the detection limit at lines 274 - 278 and 309 - 311.

4. In the legend of Figure 1 (lines 588 - 589) it is reported: "Boxes represent the median and 25th-75th percentiles, outer lines represent the 10th -90th percentiles".

Torino, March 26, 2012

Dear Editor,

We are sending the manuscript “*Estrogenic activity of an industrial wastewater effluent by means of the E-screen test and the MELN gene-reporter luciferase assay*” that we submit for possible publication on *The Science of the total environment*.

In the present study we were interested in the biological quantification of estrogenic activity in the effluent of a textile factory wastewater treatment plant (WWTP), in north-western Italy. Samples of the effluent were collected monthly before and after the tertiary treatment (ozonation) and all samples were tested to determine the total estrogenic activity by means of two in vitro tests: the *E-screen test* and the *MELN luciferase gene-reporter assay* by measuring the 17 β -estradiol equivalent quantity (EEQ). There is a positive correlation between the two tests and the results suggest that the post-ozonation effluent had lower estrogenic activity. The study points out that the environmental risk due to the input of estrogenic substances into the river via WWTP effluent is lower than the concentration at which these compounds have been reported to chronically affect the endocrine system of living organisms.

Best regards

Sincerely

Tiziana Schilirò

Ms. Ref. No.: STOTEN-D-12-00872R1

Dear Editor,

please find enclosed the new revised version of the manuscript entitled: "**Estrogenic activity of a textile industrial wastewater treatment plant effluent evaluated by the E-screen test and MELN gene-reporter luciferase assay**", hoping that it can now be accepted for publication on *The Science of the Total Environment*.

We have changed, clarified and add what the reviewer have recommended.

The notes detailing the changes to the paper and our replies to the reviewers' comments are also enclosed. We also attach a further copy of the manuscript where changes are marked in red.

We think our manuscript falls in (7) Biomonitoring area.

Best regards
Tiziana Schilirò

Response to Reviewer comment:

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Estrogenic activity of an industrial wastewater treatment plant effluent by means of the *E-screen* test and the MELN gene-reporter luciferase assay.

Highlights

1. The two *in vitro* tests are suited for estrogenic activity assessment in textile WWTP.
2. There is a significant correlation between the results of the two *in vitro* tests.
3. The estrogenic activity of the effluent is reduced by ozonation.
4. The input of estrogenic substances into the river via textile WWTP is low.

1 **Estrogenic activity of a textile industrial wastewater treatment plant effluent evaluated**
2 **by the *E-screen* test and MELN gene-reporter luciferase assay.**

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7 4 Tiziana Schilirò^{*a}, Arianna Porfido ^a, Federica Spina^b, Giovanna Cristina Varese^b, Giorgio
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26 **Abstract**

1
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4 28 wastewater treatment plant (IWWTP) in northwestern Italy. Samples of the IWWTP effluent
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43
44 44 endocrine system of living organisms.

46 **Keywords**

47 Estrogenic activity, *E-screen*, MELN, wastewater treatment plant, textile, ozonation,
48 citotoxicity.

51 **1. Introduction**

1
2 52 The potential role of endocrine-disrupting chemicals (EDCs) in the environment has been
3
4 53 discussed extensively for several years, both in the scientific community and in the broader
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6
7 54 public (Sumpter, 2008). EDCs are defined as “exogenous substances that cause adverse health
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9 55 effects in an organism, or its progeny, consequent to changes in endocrine functions”, and are
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11 56 included in the list of so-called “emerging contaminants” published by the European Union
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13 57 (EU Commission, 2001). EDCs are environmental contaminants that interfere with the
14
15 58 function of the endocrine system, and in recent years, they have become one of the major
16
17 59 subjects of environmental science research (Colborn et al., 1993; Crews et al., 2000). Recent
18
19 60 studies on EDCs in the environment have shown that these chemicals include plastic softeners
20
21 61 (bisphenol-A), detergents (4-nonylphenol or 4-octylphenol), heavy metals (cadmium), and
22
23 62 natural and synthetic compounds such as 17beta-estradiol (E2) and 17alpha-ethinyl estradiol
24
25 63 (EE2) (Ying et al., 2002; Tan et al., 2007). The scientific community has focused intensely on
26
27 64 estrogenic EDCs, which are able to interact with human estrogen receptor alpha (hER α),
28
29 65 mainly because the ligand-binding domain gap is larger than that required by E2 (Brzozowski
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31 66 et al., 1997).

32
33
34 67 EDCs are ubiquitous in the environment because of their large number of uses in residential,
35
36 68 industrial, and agricultural applications. It has been suggested that industrial and municipal
37
38 69 effluents and urban and agricultural runoff are the major sources of EDCs discharged into the
39
40 70 aquatic environment (Routledge et al., 1998; Boyd et al., 2003; Liu et al., 2009). Wastewater
41
42 71 treatment plant (WWTP) effluents likely affect reproductive processes in exposed freshwater
43
44 72 and marine organisms (Purdom et al., 1994; Orlando and Guillette, 2007). Furthermore, it has
45
46 73 been hypothesised that the increasing incidences of breast and testicular cancers in humans
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48 74 may be caused by exposure to EDCs, especially via drinking water (Carlsen et al., 1995; Safe,
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50 75 2005). For these reasons, it is important to note that surface waters are often used as a source
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76 of drinking water (Itoh et al., 2004). The presence of EDCs in the water cycle (wastewater-
77 aquatic systems-drinking water) is considered a major environmental issue. European
78 environmental regulation has prioritised the reduction of surface water pollution due to
79 municipal and industrial wastewater effluents (Directive 2000/60/EC). The current Italian
80 regulations prescribe emission limits for WWTP effluents for a wide range of chemical
81 compounds, though not specifically for EDCs (Decree Italian Law 152/2006).

82 Textile industry effluents contain high concentrations of organic and inorganic compounds
83 (Laing, 1991). Considering both the volume and the composition of the effluents, the textile
84 industry is rated as the most polluting among all industrial sectors. Textile effluents are one of
85 the most difficult-to-treat wastewaters due to their considerable suspended solids content and
86 their high concentrations of dyes, salts, additives, detergents and surfactants. The presence of
87 EDCs in textile effluents has been demonstrated (Pothitou and Voutsas, 2008; Prigione et al.,
88 2008; Shannon et al., 2008). The physical-chemical technologies used for wastewater
89 treatment, including advanced oxidation processes (i.e., the application of ozone, hydrogen
90 peroxide or ultraviolet radiation), are expensive, not always effective and often do not reduce
91 the toxicity of the effluents (Tehrani-Bagha et al., 2010; Anastasi et al., 2011).

92 In general, in a WWTP, natural and synthetic EDCs are subjected to a variety of treatment
93 processes (e.g., coagulation, sedimentation and filtration) similar to those used to inactivate
94 other compounds, but they are only partially removed. Little is known about the ultimate fate
95 of endocrine disruptors, particularly after disinfection steps. However, recent studies on the
96 effects of chlorination (Deborde et al., 2004; Lee et al., 2004; Schilirò et al., 2009) and
97 ozonation (Irmak et al., 2005; Bila et al., 2007; Stalter et al., 2011) on endocrine disruptors
98 suggested that they may be degraded via oxidation (Silva et al., 2012).

99 Several screening programs using a variety of chemical analyses, as well as *in vitro* and *in*
100 *vivo* bioassays, have been implemented to assess the potential hazard of EDCs in aquatic

101 environments (Campbell et al., 2006). Considering the large number of EDCs that may be
102 present in a complex environmental sample, target chemical analyses are not sufficient to
103 comprehensively define its EDC content. Furthermore, the total biological effects of the
104 whole sample cannot be determined in these analyses (Nelson et al., 2007; Bicchi et al.,
105 2009). In contrast, several *in vitro* bioassays based on the interaction between the EDCs and
106 estrogenic receptors can be used to determine the total estrogenic EDC activity of an
107 environmental sample. These assays allow the evaluation of additive, synergistic and
108 antagonistic effects (Balaguer et al., 1999; Korner et al., 1999; Leusch et al., 2010).
109 Moreover, these evaluations can be made more comprehensive with the parallel use or
110 combination of two or more tests (Leusch et al., 2010).

111 In this study, the total estrogenic activity in the effluent of an industrial wastewater treatment
112 plant (IWWTP) in northwestern Italy was measured. Samples of the IWWTP effluent were
113 collected from a textile factory each month from November 2009 to July 2010. After solid
114 phase extraction, all samples were subjected to two *in vitro* tests to measure the 17beta-
115 estradiol equivalent quantity (EEQ): the human breast cancer cell line (MCF-7 BUS)
116 proliferation test or, *E-screen* test, and the luciferase-transfected human breast cancer cell line
117 (MELN) gene-reporter assay. The IWWTP effluent was evaluated before and after tertiary
118 treatment (ozonation) to assess the potential impact of disinfection on estrogenic activity. The
119 results of the two *in vitro* tests were compared.

121 2. Materials and Methods

122 2.1 Characteristics and sampling of industrial wastewater treatment plant (IWWTP) 123 effluents.

124 The effluents monitored in this study were sampled from a small IWWTP associated with a
125 textile factory in northwestern Italy. The mean treated wastewater flow is approximately

126 3,000 m³/day, mainly released by cotton dyeing processes. The dyes are synthetic organic
127 indanthrenes, derived from anthraquinones, that are particularly stable in response to light,
128 weathering and the detergents/disinfectants used for dyeing various fibres. The plant has
129 separate water and sludge treatment lines; the former includes primary (chemico-physical),
130 secondary (activated sludge) and tertiary processes (ozonation). Twenty-four hour composite
131 samples of the final IWWTP effluents were sampled after the secondary treatment (pre-O₃)
132 and after the tertiary treatment (post-O₃) to evaluate the effects of ozonation on estrogenicity.
133 Table 1 describes the physico-chemical characteristics of the effluent samples and the relative
134 Italian threshold limit values (Decree Italian Law 4/2008). The ozonation treatment was
135 carried out in columns (0.2 m in diameter and 4.5 m in height) by adding in countercurrent
136 (effluent flow rate of 60 L/hr) 30 ppm of ozone. Samples were taken on 7 different sampling
137 dates from November 2009 to July 2010. The samples (2 L) were stored in brown glass flasks
138 at 4°C. Two-litre aliquots were extracted for the *E-screen* test and the MELN gene-reporter
139 luciferase assay.

2.2 Extraction of effluent samples

141 The extraction of effluent samples was evaluated using a previously described method
142 (Schilirò et al., 2009). Briefly, solid phase extraction was performed on columns with 1 g
143 polystyrene copolymer resin ENV and a 6-mL reservoir (Varian, Inc. Agilent technologies,
144 USA) (Kinnberg K, 2003). Two-litre aliquots of each sample were extracted. Samples were
145 first equilibrated to room temperature; then, methanol was added, the pH was adjusted to 2.5,
146 and NaCl was added to achieve a conductivity of 8500 µS. Samples were then drawn through
147 the column after its activation. Elution was performed with 5 mL acetone. Each extract was
148 evaporated to 1 mL under a stream of nitrogen. A 100 µL aliquot of dimethylsulfoxide
149 (DMSO 99.5%) was added to the extract, and the acetone was then completely removed under
150 the nitrogen stream. The extracts were stored in glass vials at 4°C until evaluated in the *E-*

151 *screen* test and the MELN gene-reporter luciferase assay. Two-litre Milli-Q water were
152 extracted and analyzed as a QC laboratory sample.

153 *2.3 Sample preparation for bioassays*

154 Each sample extract was mixed with 9.90 mL steroid-free experimental medium, and each
155 sample was then homogenised for approximately 2 min and filtered through a 0.22 μ m
156 Millex-GV filter (Millipore). These stock solutions containing 1% v/v DMSO were stored in
157 sterile glass vials at 4°C. They were diluted 10- to 100,000-fold (0.05 to 500 L final volume)
158 with steroid-free experimental medium in sterile glass vials. This ensured that the maximum
159 solvent concentration in the culture medium did not exceed 0.1%, a concentration that was
160 found to have no effect on cell viability.

161 *2.4 Cell lines and culture conditions*

162 Estrogen-sensitive human MCF-7 BUS breast cancer cells were kindly provided by Dr. A.M.
163 Soto and Dr. C. Sonnenschein (Tufts University School of Medicine, Boston, Massachusetts,
164 USA) and cultivated in Dulbecco's modified Eagle's medium (DMEM) with 15 mg/L phenol
165 red, 10% foetal calf serum (FCS), 2% L-glutamine 200mM, 2% HEPES buffer 1M, 1%
166 sodium pyruvate 100mM and 1% penicillin-streptomycin 10 mg/mL, at 37°C in an
167 atmosphere containing 5% carbon dioxide and 95% air under saturating humidity. A stock
168 solution of 1 mM 17- β -estradiol (E2) was prepared in ethanol, stored at -20°C and then
169 diluted to the desired concentration in steroid-free experimental medium.

170 MELN cells, provided by Dr. P. Balaguer (INSERM, Montpellier, France), are MCF-7 cells
171 stably transfected with the estrogen-responsive gene (ERE- β Glob-Luc-SVNeo) via integrated
172 plasmid. These plasmids contain both an antibiotic resistance selection gene (SVNeo) and the
173 estrogen-responsive elements to which the estrogen receptor-ligand complex can bind, thus
174 inducing the transcription of the luciferase reporter gene (Berckmans et al., 2007). Therefore,
175 the luciferase activity in these cells is proportional to the concentration of estrogenic

176 compounds (Hernandez-Raquet et al., 2007). MELN cells were cultured in Dulbecco's
177 Modified Eagle Medium Nutrient Mixture F12 Ham (DMEM-F12) with phenol red,
178 supplemented with 5% foetal calf serum (FCS), 2% 200 mM L-glutamine, 1%
179 penicillin/streptomycin and 1 mg/ml G418 sulphate. The cells were maintained in an
180 incubator at 37°C, a relative humidity of 95% and a CO₂ concentration of 5%. The cells were
181 subcultured once per week, and the medium was refreshed between passages. Cells from
182 passage number 4 to passage number 15 were used for experiments. Cells were regularly
183 checked for mycoplasma infection to guarantee that the experiments were performed with
184 mycoplasma-free cells and to comply with the guidelines for good cell culture practice
185 (GCCP). Unless otherwise specified, all chemicals and materials for cell culture were
186 obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.5 *E-screen test*

The *E-screen* test was carried out as initially described by Korner et al. (1999) and modified
by Schilirò et al. (2009). Briefly, subconfluent MCF-7 BUS cells were trypsinised and
resuspended in the steroid-free experimental medium, which consisted of phenol-red-free
DMEM supplemented with 5% stripped FCS, 2% L-glutamine 200mM, 2% HEPES buffer
1M, 1% sodium pyruvate 100mM and 1% penicillin-streptomycin 10 mg/mL. Cells were
seeded into 24-well plates at a density of 30000 cells/well. After 24 hours, the medium was
replaced with experimental medium containing one of five dilutions of water extracts. Each
dilution was tested in six replicates per assay. Moreover, one dilution (100-fold) of each
effluent sample was tested in combination with 5 nM of the antiestrogen tamoxifen (Tam) and
with 0.1 nM E2. Six wells without hormones were used as negative controls, and E2, in five
concentrations between 1 pM and 10 nM, was used as the positive control in each assay. Each
E-screen assay was also performed on a QC laboratory sample (treated with the same method

200 of samples). The assays were completed after six days by determining the absorbance (595
 1
 2 201 nm) in each well after crystal violet staining.

3
 4 202 The proliferative effect (PE) of a sample is the ratio between the highest cell number achieved
 5
 6
 7 203 with the sample or E2 and the cell number of the negative control:

8
 9
 10 204 $(1) PE = (\text{max cell number})_{\text{sample}} / (\text{cell number})_{\text{negative control}}$.

11
 12 205 The estrogenic activity of a sample is evaluated by determining the relative efficacy, called
 13
 14 206 the relative PE (RPE%). The RPE compares the maximum proliferation induced by a sample
 15
 16
 17 207 with that induced by E2:

18
 19 208 $(2) RPE \% = [(PE-1)_{\text{sample}} / (PE-1)_{\text{E2}}] \times 100$.

20
 21
 22 209 Full agonistic activity, $RPE \geq 100\%$, can be distinguished from partial agonistic activity, in
 23
 24 210 which RPE is less than 100% (Soto, 1995).

25
 26
 27 211 Relative potency, called estradiol equivalency quantity (EEQ) is thus calculated as:

28
 29 212 $(3) EEQ = (EC50)_{\text{E2}} / (EC50)_{\text{sample}}$.

30
 31 213 The EC50 value for the *E-screen* test (concentration at which 50% of PE is achieved) was
 32
 33
 34 214 calculated with a probit regression (SPSS, Chicago, IL). The PE and EC50 values of each
 35
 36 215 sample were calculated from the mean dose–response curves established from each
 37
 38
 39 216 experiment. The EEQ, expressed in ng/L, is defined as the total concentration of estrogenic
 40
 41 217 active compounds in an environmental sample normalised to the natural estrogen 17- β -
 42
 43
 44 218 estradiol.

45 46 219 *2.6 MELN gene-reporter luciferase assay.*

47
 48
 49 220 MELN gene- reporter luciferase assay has been widely used for the detection of estrogenic
 50
 51 221 activity in complex environmental samples (Cargouët et al., 2007; Combalbert et al., 2012;
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 53
 54 222 Fenet et al., 2003; Hernandez-Raquet et al., 2007; Jujan et al., 2009; Kinani et al., 2010;).
 55
 56 223 The test was carried out as described by Balaguer et al. (1999).
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224 Because phenol red and FCS exhibit estrogenic activity, *in vitro* experiments were carried out
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2 225 in DMEM F12 without phenol red, supplemented with 5% dextran-coated charcoal-treated
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4 226 foetal calf serum (DCC-FCS), 2% L-glutamine 200 mM and 1% antibiotics
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6
7 227 (penicillin/streptomycin). To adapt the cells to DCC-FCS, the growth medium was replaced
8
9 228 with fresh test medium 3 days prior to the experiment. Cells were then harvested and seeded
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11 229 in 96-well plates with a flat, clear bottom (Corning) at a density of 40000 cells/well in 100 μ l
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14 230 of DCC-FCS per well. After 24 hours, the test medium was removed, and 100 μ l of each
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17 231 sample dilution was added to three replica wells. Cells were treated with samples for 20 h.
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19 232 Negative controls, without hormones, and positive controls, E2 in concentration between 1
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21
22 233 pM and 10 nM, were included in each assay. One dilution (100-fold) of each effluent sample
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24 234 was tested together with 5 μ M antiestrogen tamoxifen (Tam) and with 0.1 nM E2. Each
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26 235 *MELN gene-reporter luciferase assay* was also performed on a QC laboratory sample (treated
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29 236 with the same method of samples). All experiments were performed in triplicate.

31 237 *2.6.1 Luciferase activity measurement in MELN cells.*

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34 238 We used the One-Glo Luciferase Assay System (Promega, USA) to measure luciferase
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36 239 activity according to manufacturer's instructions. Briefly, at the end of the incubation, 100 μ l
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39 240 of One-Glo Reagent (containing fluoroluciferin) was added to each well and mixed for
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41 241 optimal consistency. After at least 3 minutes, to allow complete cell lysis, and within 30
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43
44 242 minutes of reagent addition, luminescence was measured by a luminometer (Tecan, Infinite
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46 243 M200 PRO). The luciferase activity of the MELN cells relative to the positive control E2 was
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48
49 244 represented as transactivation % (TRANS %): the maximum increase in luciferase gene
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51 245 expression triggered by estrogenic compounds present in the samples. The induction of
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54 246 luciferase activity was expressed as a percentage, and the 100% value was obtained in the
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56 247 presence of E2 (Balaguer et al., 1999; Fenet et al., 2003). The estrogenic activity was
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248 expressed as 17-beta estradiol equivalent estrogenic activity (EEQ) in ng/L. EEQ was thus
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2 249 calculated as $(EC50)E2/(EC50)_{sample}$.

250 *2.6.2 Cytotoxicity assessment in MELN cells.*

251 The Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, cat. no. 9200) is a fluorescence-
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9 252 based assay that use membrane integrity changes to measure cell viability or cytotoxicity in
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11 253 conjunction with luciferase measurements on the same test plates (Berckmans et al., 2007).
12
13 254 This assay technology simultaneously measures two distinct protease activities, with rapid
14
15 255 catalytic cleavage rates, as markers of cell viability or cytotoxicity.
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18 256 The cytotoxicity test was applied to dilutions 1:10 and 1:100 (equal to 5 L and 0.5 L dilution
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21 257 volumes) of the pre- and post-ozonation effluents.
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24 258 At the end of incubation, 100 μ l aliquots of reagent were added to all wells, mixed and
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26 259 allowed to incubate for at least 30 minutes at 37°C. Fluorescence was determined at
27
28 260 excitation/emission wavelengths of 360 nm/460 nm for cell viability and 485 nm/528 nm for
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30 261 cytotoxicity using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT).
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33 262 The mean values and standard deviations of replicate wells and the mean fold increase of
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35 263 cytotoxicity relative to the negative control were calculated.
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38 264 *2.7 Statistical analyses.*

39 265 Data were analysed by means of a probit regression analysis (EC50), Spearman's test
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41 266 (correlation) and t-test (means comparison) using SPSS 18.0 (SPSS for Windows, Chicago,
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43 267 IL, USA).
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49 269 **3. Results and Discussion**

50 270 *3.1 Estrogenic activity in IWWTP effluent samples as measured by E-screen test.*

51 271 The mean EC50 value of E2 for the *E-screen* was 15.24 ± 13.73 ng/L (56.44 ± 50.83 pM);
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53 272 EC50 values were calculated from the control curves obtained from each of the bioassays.
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273 Maximum cell proliferation was generally induced by 0.1 nM E2. The detection limit of the
1
2 274 *E-screen* could be defined as the concentration of a single compound or a sample inducing a
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4 275 cell proliferation significantly higher than the hormone-free negative control; the treatment
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7 276 with 17beta-estradiol showed a detection limit of 1 pM (Körner et al. 1999; Schilirò et al.,
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9
10 277 2004). The proliferative response of the samples was dose dependent; the 1:100 dilution of
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12 278 the samples (equal to a 0.5 L dilution volume) always stimulated the maximum proliferative
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14 279 response. The QC laboratory sample did not induce any significant cell proliferation. To
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16
17 280 verify that the cell proliferation induced by estrogenic activity was mediated by ER, we
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19 281 observed that co-incubation with Tam inhibited the proliferative response ($28 \pm 23 \%$), while
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22 282 co-incubation with E2 led to a greater proliferative response ($114 \pm 39 \%$). The difference was
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24 283 statistically significant in both cases (t-tests, $p < 0.05$).

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27 284 Estrogenic activity, expressed as EEQ, was detected in all but the sixth sample and ranged
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29 285 from 0.10 to 4.73 ng/L (Table 2). The proliferative effect of the different effluent samples on
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31
32 286 MCF-7 BUS cells relative to the positive control E2 is represented as RPE % in Table 2. The
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34 287 RPE of the effluent both before and after ozonation generally showed partial agonist activity
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36 288 (RPE < 100%) ranging between 15 to 90 %. The EEQs of the effluent extracts, determined in
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39 289 the *E-screen* test, are reported in Figure 1.

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42 290 The highest mean EEQ was found in the second pre-ozonation sample (4.73 ± 2.42 ng/L), and
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44 291 the lowest mean EEQ was found in the last post-ozonation sample (0.10 ± 0.13 ng/L). The
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46 292 mean EEQ values were 2.35 ± 1.68 ng/L pre-ozonation and 0.72 ± 0.58 ng/L post-ozonation.

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49 293 The average reduction in the estrogenic activity of IWWTP effluent from pre- to post-
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52 294 ozonation was $67 \pm 26 \%$. Despite the low number of analysed samples, the difference
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54 295 between EEQ values before and after ozonation was statistically significant (simple paired t-
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57 296 test, $p = 0.038$), suggesting that the ozonation process significantly reduced the estrogenic
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59 297 activity of the IWWTP effluent.

298 The *in vitro E-screen* test can be used to screen a large number of compounds and integrate
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2 299 the effects of chemicals that may not be measured in an analytical screen (Vega-Lopez, 2007).
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4 300 It has been studied extensively, and no false positives or false negatives have yet been found;
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7 301 it is also one of the most sensitive of all published assays (Soto et al., 2006; Vanparys et al.,
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9 302 2010). The *E-screen* has been shown to be appropriate for the determination of estrogenic
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11 303 activity in environmental extracts from IWWTP effluent samples.

14 304 3.2 Estrogenic activity in IWWTP effluent samples by MELN gene-reporter luciferase 15 16 305 assay

17 306 The EC50 values of E2 in the MELN gene-reporter luciferase assay were calculated from
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19 307 control curves obtained from each bioassay, and the mean EC50 value was 1.76 ± 0.87 ng/L
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22 308 (6.52 ± 3.22 pM). Maximum luciferase activity was generally induced by 0.1 nM E2. The
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24 309 stable transfectants MELN used in this study exhibited good responsiveness following
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27 310 treatment to 17beta-estradiol with a detection limit of 1 pM (Balaguer et al., 1999). The
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29 311 luciferase activity of the samples was dose dependent, and the 1:100 dilution (equal to a 0.5 L
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32 312 dilution volume) always stimulated the maximum luciferase activity. The QC laboratory
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34 313 sample did not induce any significant luciferase activity. To confirm that the luciferase
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37 314 activity was mediated by the ER, we observed that co-incubation with Tam led to an
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39 315 inhibition of the proliferative response (51 ± 29 %), while co-incubation with E2 led to a
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42 316 greater proliferative response (113 ± 34 %). The difference was statistically significant in both
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45 317 cases (t-tests, $p < 0.05$). Figure 2 shows the MELN luciferase activity of E2 and of the post-
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47 318 ozonation effluents expressed in dose-response curves and compared with the negative
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49
50 319 control.

51 320 Estrogenic activity was detected in all but the sixth sample and ranged from 0.14 to 8.50 EEQ
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54 321 (ng/L) (Table 2). The luciferase activity of the different effluent samples in the MELN cells
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57 322 relative to the positive control E2 is represented as TRANS % in Table 2. The TRANS % of
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323 the effluent before and after ozonation ranged between 27 to 62 %. The EEQs of the effluent
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2 324 extracts, determined in the MELN gene-reporter luciferase assay, are reported in Figure 1.

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5 325 The highest mean EEQ was found in the first pre-ozonation sample (8.50 ± 4.36 ng/L), and
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7 326 the lowest mean EEQ was found in the second post-ozonation sample (0.14 ± 0.05 ng/L). A
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10 327 lower mean value was found for the effluents post-ozonation: the mean EEQ values were 4.18
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12 328 ± 3.54 ng/L pre-ozonation and 2.53 ± 2.48 ng/L post-ozonation; this difference was
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14
15 329 statistically significant (simple paired t-test, $p = 0.048$). The average reduction of estrogenic
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17 330 activity of IWWTP effluent after ozonation was 52 ± 27 %.

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20 331 The *in vitro* MELN gene-reporter luciferase assay can rapidly screen a large number of
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22 332 chemicals, and it is transferable, robust and reproducible, enabling the ranking of chemical
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25 333 compounds from strong to weak affinity for the estrogen receptor (Witters et al, 2010). This
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27 334 assay has been shown to be appropriate for the determination of estrogenicity in
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30 335 environmental extracts from IWWTP effluent samples.

31 32 336 *3.3 Cytotoxicity of IWWTP effluent samples in MELN cells.*

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34 337 The 1:10 dilution of the effluent pre-and post-ozonation was shown to decrease cell
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37 338 proliferation compared to negative controls, in terms of PE, in both assays. The sample, which
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40 339 is less diluted, might be able to mask the real estrogenic activity of the compounds present in
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42 340 it. To determine whether the effluent might have a toxic effect on the cells, 1:10 and 1:100
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44 341 dilutions (equal to 5 L and 0.5 L dilution volume) were tested for cytotoxicity (Table 3).

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47 342 The 1:10 mean fold increase of cytotoxicity relative to the negative control was 50.3 ± 30.0
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50 343 for pre-ozonation effluents and 6.3 ± 6.0 for post-ozonation effluents (t-tests, $p < 0.01$ and $p <$
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52 344 0.05 , respectively). The mean fold increase of cytotoxicity produced by the 1:100 dilution
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54 345 compared to the negative control was 1.2 ± 0.8 for the pre-ozonation effluent and 1.0 ± 1.1 for
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57 346 the post-ozonation effluent (t-tests, $p > 0.05$). The sample without estrogenic activity ($n^{\circ}6$) did
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347 not exhibit a significantly higher cytotoxicity; therefore, the absence of estrogenicity can not
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2 348 be ascribed to the toxicity of the sample.
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4 349 *3.4 Comparison of estrogenic activity in the E-screen test and in the MELN gene-* 5 6 7 350 *reporter luciferase assay* 8

9 351 A comparison of the results obtained in these two assays highlights the non-significance of
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11 352 differences in average EEQs (t-test, $p > 0.05$). The correlation between the EEQs obtained
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13 353 with the MELN gene-reporter luciferase assay and with the *E-screen* was positive and
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15 354 significant (Rho S = 0.650 and $p = 0.022$). The mean EC50 values relative to E2 were $15.24 \pm$
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17 355 13.73 ng/L for the *E-screen* and 1.76 ± 0.87 ng/L for the MELN gene-reporter luciferase
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19 356 assay; these values were significantly different (t-test, $p = 0.015$). The MELN gene-reporter
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21 357 luciferase assay generally showed higher EEQ mean values.
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25 358 These differences could be partially explained by the end-points of the two tests: the *E-screen*
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27 359 is based on a binding mechanism that causes proliferation as a cellular response, which could
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29 360 be affected by other external factors, while the MELN assay is receptor specific. Another
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31 361 important difference between the two tests is in the assay duration; stimulation with the test
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33 362 compounds lasts for 16-20 hours in the MELN assay but 120 hours for the *E-screen* (Soto et
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35 363 al., 2006; Witters et al, 2010).
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44 365 **4. Conclusions**

45 366 In general, the *E-screen* test and the MELN gene-reporter luciferase assay are well suited for
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47 367 the determination of estrogenic activity in environmental matrices; in this specific study, they
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49 368 were used to evaluate estrogenic activity in wastewaters from a textile factory, before and
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51 369 after treatment with ozone. The two *in vitro* tests are particularly suitable for environmental
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53 370 monitoring because of their sensitivity, speed, reading and low cost. The estrogenicity values
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55 371 in the final effluent (post O₃) in the present work were similar to results reported in the
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372 literature for other IWWTP effluents (Korner et al., 1999; Onda et al., 2002; Vethaak et al.,
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2 373 2005; Tan et al., 2007; Salste et al., 2007). Our study suggests that the input of estrogenic
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4 374 substances into the river via IWWTP is low (EEQ range: 0.72 – 2.53 ng/L) and that the
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7 375 environmental risk could be even lower due to the dilution effect connected with discharge
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10 376 and the self-depuration capacity of the receiving river. In a previous study, this reduction was
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12 377 approximately one order of magnitude for EEQs (Schilirò et al., 2009), resulting in
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14 378 concentrations lower than those at which these compounds have been reported to chronically
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17 379 affect the endocrine systems of living organisms (approximately 1-10 ng/L; Lopez de Alda &
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19 380 Barcelo, 2001). The analysed effluent shows lower EEQs values relative to a municipal
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22 381 effluent (Fenet et al., 2003; Pothitou and Voutsas, 2008; Pereira et al., 2011). This difference
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24 382 in the estrogenic activity of urban and industrial wastewater is certainly attributable to the
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27 383 presence of different types of substances; municipal wastewater contains more natural EDCs,
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29 384 such as E2 and EE2, that have strong estrogenic power even at very low concentrations, while
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32 385 industrial wastewater typically contains more synthetic EDCs that are weakly estrogenic
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34 386 relative to natural EDCs (Soto et al., 1995; Pereira et al., 2011).

36 387 Ozonation is considered an economically feasible option for the advanced treatment of
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39 388 WWTP effluents (Joss et al., 2008). It is a very effective method for the degradation of
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41 389 persistent organic dyes from colored textile wastewater (Tehrani-Bagha et al., 2010). In
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44 390 general, ozonation and other oxidation processes seem to be the best alternatives for treating
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46 391 WWTP effluents containing estrogens (Pereira et al., 2011). Some authors have noted that the
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49 392 decrease in EDCs may be due to the oxidation effects of ozonation (Lee, 2004; Larcher et al.,
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51 393 2012). To our knowledge, few reports in the literature describe estrogenic activity in textile
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54 394 IWWTP. This work shows that this type of effluent does have estrogenic activity and that
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56 395 ozonation decreases its overall estrogenic effects. However, ozonation is known to lead to the
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58 396 formation of transformation products that have largely not been identified to date, and there
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397 are concerns about their potential impact on the environment and human health (Benner and
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2 398 Ternes, 2009; Radjenovic et al., 2009; Dodd et al., 2010; Stalter et al., 2010, 2011; Anastasi et
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4 399 al., 2011; Reungoat et al., 2012). This highlights the need to evaluate the presence of
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7 400 disinfection by-products after water treatment has been performed.

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9 401 The identification and removal of EDC pollutants from IWWTP effluents may solve many of
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11 402 the apparent endocrine disruption problems observed in aquatic environments, in addition to
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13 403 providing a cleaner source of drinking water. Further investigations should be carried out to
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16 404 identify the level of ozone appropriate to minimise both the estrogenic activity and the
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19 405 toxicity of IWWTP textile effluents.

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32
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35
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37
38
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42 43 415 **6. References**

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36 583 **Figure captions**
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41 585 **Figure 1.** Estradiol equivalency quantity (EEQ ng/L) of the IWWTP effluent extracts after the
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43 586 secondary treatment (Pre ozonation) and after the tertiary treatment (Post ozonation) by
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45 587 means of *E-Screen* test and MELN gene-reporter luciferase assay. Boxes represent the median
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47 588 and 25th – 75th percentiles, outer lines represent the 10th -90th percentiles.
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53 590 **Figure 2.** MELN luciferase activity of 17-beta-estradiol (E2) and of post ozonation effluents
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55 591 (examples: n° 5 dotted bars and n° 7 striped bars) expressed with dose-response curves and
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57 592 compared with the negative control.
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- 1 **Table 1.** Physico-chemical parameters of the effluents after secondary treatment (Pre-O₃) and
 2 after tertiary treatment (Post-O₃) and Italian threshold limit values (nd: not detected).

Parameters	Pre O ₃	Post O ₃	Italian threshold limits values*
pH	8.3 – 9.4	8.4 – 9.2	5.5 < x < 9.5
Colour	Often detectable after 1:20 dilution	nd, after 1:20 dilution	nd, after 1:20 dilution
Suspended solids (mg/L)	18 – 58.8	17 - 27	< 80
COD (mg/L)	53.3 - 81.0	33.7 - 89.3	< 160
BOD (mg/L)	9 - 20	8 - 14	< 40
Cl ⁻ (mg/L)	235.6 - 925	795 - 1102	< 1200
Sulphites (mg/L)	0.44 - 0.72	< 0.2	< 1
Sulfates (mg/L)	269 - 948	479 - 561	< 1000
NH ₄ (mg/L)	0.05 - 1.025	< 0.1	< 15
Nitrites (mg/L)	0.1 - 0.2	< 0.02	< 0.6
Nitrates (mg/L)	1.04 - 1.4	7.1 - 11.9	< 20
Total tensioactives (mg/L)	1.4 - 1.5	< 0.2	< 2

3 *Decree Italian Law 4/2008, reference values for discharge to surface waters.
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- 6 **Table 2.** Mean estrogenic activity of the IWWTP effluent extracts after secondary treatment
 7 (Pre-O₃) and after tertiary treatment (Post-O₃) in the *E-screen* test and MELN gene-reporter
 8 luciferase assay (nd: not detected).

N° sampling	<i>E-screen</i> test EEQ (ng/L)	<i>E-screen</i> test RPE%	<i>MELN</i> assay EEQ (ng/L)	<i>MELN</i> assay TRANS %
Pre O₃				
1	1.66 ± 0.85	37	8.50 ± 4.36	29
2	4.73 ± 2.42	39	1.25 ± 0.37	42
3	1.78 ± 2.37	65	7.38 ± 0.87	27
4	4.17 ± 1.38	74	6.12 ± 3.34	58
5	0.79 ± 0.30	6	1.04 ± 0.39	46
6	nd	nd	nd	nd
7	0.99 ± 0.27	45	0.78 ± 0.28	37
Post O₃				
1	1.34 ± 0.69	32	3.92 ± 2.01	56
2	0.73 ± 0.38	21	0.14 ± 0.05	32
3	0.35 ± 0.47	52	5.33 ± 1.63	44
4	1.50 ± 0.20	90	5.03 ± 1.65	62
5	0.28 ± 0.10	26	0.38 ± 0.14	39
6	nd	nd	nd	nd
7	0.10 ± 0.13	15	0.41 ± 0.20	42

10 **Table 3.** MELN cytotoxicity and cell viability of the IWWTP effluent extracts (1:10 and
 11 1:100 dilutions) after secondary treatment (Pre-O₃) and after tertiary treatment (Post-O₃).

N° sampling	<i>Cytotoxicity</i> fold increase		<i>Cell viability</i> fold increase	
	1:10	1:100	1:10	1:100
Pre O₃				
1	36.2	1.0	0.6	1.6
2	89.4	1.8	0.3	1.3
3	35.3	1.0	0.5	1.5
4	26.7	1.0	0.7	1.4
5	75.4	1.7	0.5	1.5
6	71.6	0.9	0.4	1.5
7	17.3	1.2	0.6	1.3
Post O₃				
1	1.5	0.5	1.3	1.8
2	18.5	0.5	0.7	1.6
3	4.6	0.5	1.0	1.8
4	1.6	0.6	1.4	1.8
5	7.4	0.7	1.0	1.8
6	8.1	0.6	0.9	1.5
7	2.2	3.4	1.3	1.1

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Figure 1.2.
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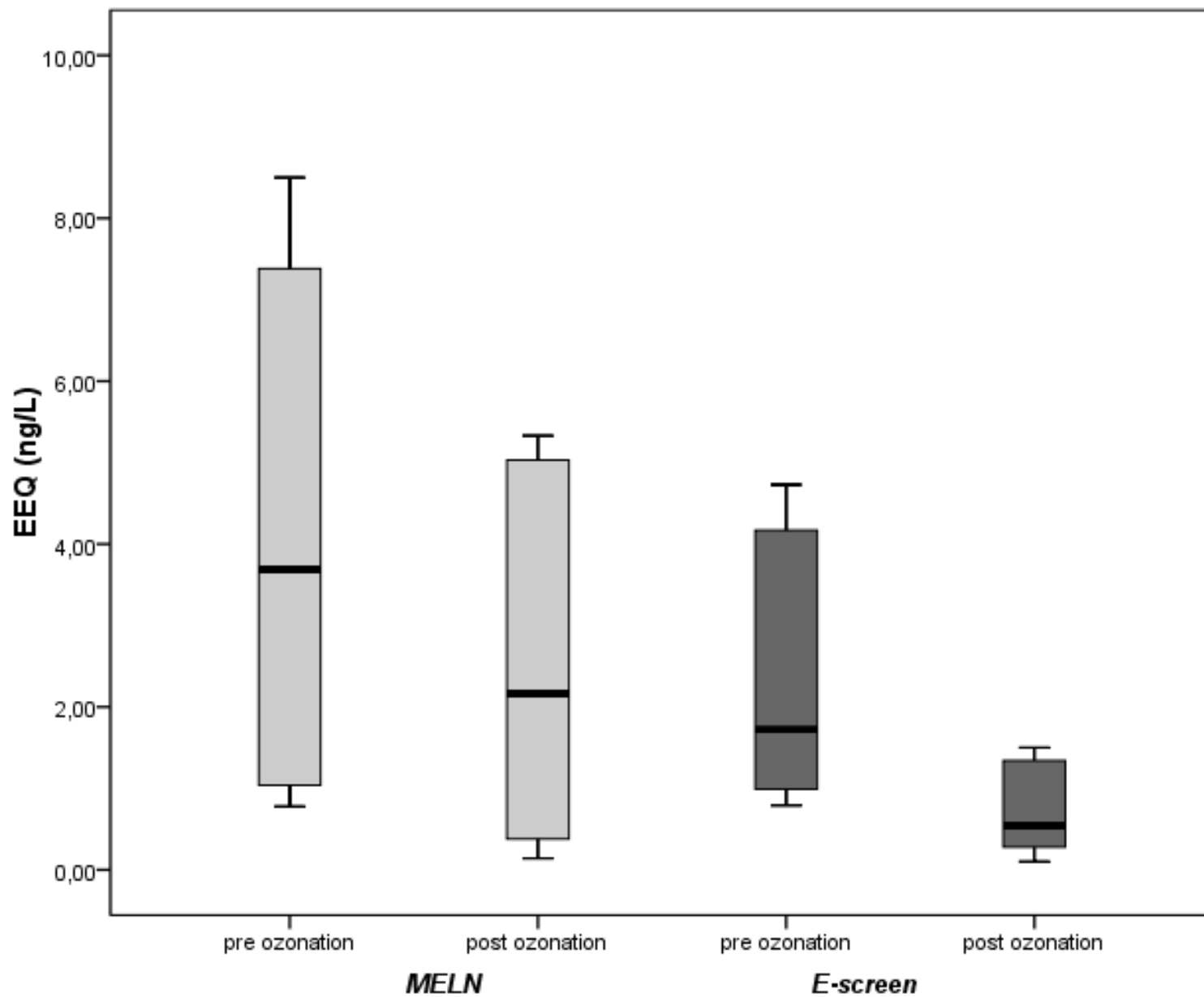
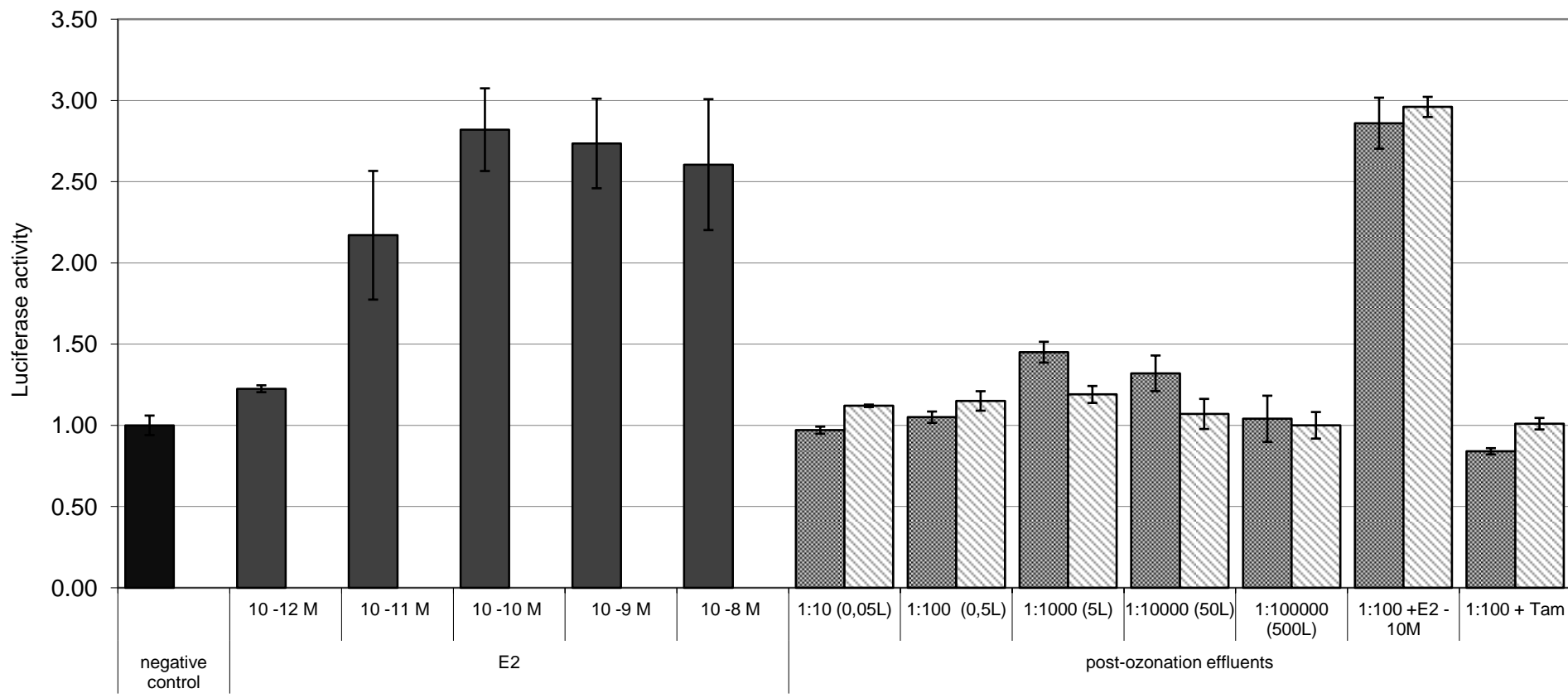


Figure 2.



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